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DISSERTATION

Different susceptibility of acutely injured and chronically epileptic brain tissue to induction of cortical spreading depolarization and seizures

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1. Preface

The present short dissertation has the aim to summarize three relevant and independent publications in which I participated during my PhD thesis (Maslarova *et al.* 2011; Lapilover *et al.* 2012; Maslarova *et al* 2013), with particular focus on my direct contribution. The dissertation follows the guidelines of the "Publication-based Thesis" within the context of the "International Graduate Program Medical Neurosciences" at the Charité–Universitätsmedizin Berlin. Background information, methodological details as well as parts of results, figures and discussion had to be shortened due to space limitations, but can be found in the respective publications, which are inserted in their entire form in section 10 of this thesis.

2. Zusammenfassung

Epileptische Entladungen und kortikale „spreading depolarizations“ (CSDs) sind zwei Muster elektrophysiologischer Aktivität, die sowohl im gesunden als auch im verletzten Gehirngewebe untersucht werden können. Beide können von denselben pathogenen Zuständen ausgelöst werden, u.a. Schlaganfall, Trauma oder Fieber. All diese Zustände sind charakterisiert durch eine Beeinträchtigung der Blut-Hirn Schranke und daher durch Austreten von Serum Proteinen wie Albumin und Immunfaktoren ins Interstitium. Zudem werden die Ionenkonzentrationen im Liquor an Serumwerte angepasst. Während Auftreten von CSDs die Akutprognose nach Gehirnschaden verschlechtert, deuten epileptische Entladungen auf eine mögliche spätere Entwicklung einer chronischen Epilepsie nach einer Latenzphase, während der Anpassungs- und Reorganisationsprozesse stattfinden. In meiner Doktorarbeit, habe ich den Zusammenhang zwischen CSD, akuter und chronischer Iktogenese und Blut-Hirn –Schrankenstörung untersucht. Anhand von elektrophysiologischen Messungen und immunohistochemischen Färbungen in temporo-hippokampalen Schnittpräparaten, konnte ich zeigen, dass CSDs und epileptische Entladungen sowohl im akut beschädigten als auch im chronisch epileptischen Gewebe zusammen auftreten können. Allerdings ist chronisch epileptisches Gewebe widerstandsfähiger gegen Mittel, die als potente Induktoren epileptischer Anfällen und/ oder CSDs im gesunden Gehirn gelten. Darüber hinaus können Stoffe, die im naiven Gewebe harmlos sind oder sogar eine physiologische Funktion ausüben (wie z.B. der Neurotransmitter Acetylcholin), in chronisch epileptischem Gewebe iktogen wirken. Demzufolge ist Übererregbarkeit nach akuter Verletzung und in chronisch epileptischem Gewebe durch unterschiedliche Mechanismen verursacht und sollte dementsprechend spezifisch therapiert werden.

Abstract

Epileptic seizures and cortical spreading depolarization (CSD) are two patterns of electrophysiological activity that can be studied in the healthy and in the injured brain. They can be triggered by the same pathologies, such as stroke, traumatic brain injury and fever, with disruption of the blood brain barrier as a common hallmark. The latter is associated with entry of serum components into the brain interstitial space, such as albumin and immune factors, and a shift of extracellular ion concentrations towards serum values. While CSD can acutely deteriorate patients’ outcome, hyperexcitability of the tissue is potentially indicative for the development of chronic seizures. Chronic epilepsy is usually preceded by an initial silent period, during which reorganization processes take place. In my thesis, I have examined the relationship between CSD, acute and chronic tissue excitability and blood brain barrier disruption, using electrophysiological recordings from acute temporo-hippocampal slices and immunocytochemistry stains of the temporo-hippocampal formation. The main findings of the work are that CSD and epileptic seizures can coexist in both acute brain injury and chronic epilepsy. However chronic epileptic tissue is more resistant to agents, which have proven to be potent inducers of epileptic seizures and/or CSDs in the healthy brain. Moreover, agents, which are harmless or even play a physiological function in the healthy brain, can become ictogenic in the chronically epileptic brain, as is the case with the neurotransmitter acetylcholine. These results provide evidence that hyperexcitability in acute brain injury and chronic epilepsy are driven by different mechanisms and therefore distinct treatment strategies should be considered.

3. Introduction and Aims

Epileptic seizures are often observed after acute brain injury such as stroke (Camilo and Goldstein, 2004; Jungehulsing et al., 2013), subarachnoid hemorrhage (Dreier et al., 2012), traumatic brain injury (TBI)(Bolkvadze and Pitkänen, 2012), and fever in childhood (Dubé et al., 2007; McClelland et al., 2011) and might indicate risk for progression to chronic epilepsy. Usually, the initial event is followed by a latent phase, during which reorganization processes take place and eventually lead to the development of recurrent seizures. Reorganization might include cell loss, fiber sprouting and changes in expression of ion channels and receptors, associated with increased excitability, such as in voltage-gated ion channels (Bernard et al., 2004; Ellerkmann et al., 2003), and in the glutamatergic, GABAergic (Ferando and Mody, 2012; Maglóczky and Freund, 2005; Tolner et al., 2007) and cholinergic system (Friedman et al., 2007; Zimmerman et al., 2008).

Not all patients develop epilepsy after an initial injury. In the case of stroke, up to 8.2% of patients develop chronic epilepsy (Jungehulsing et al., 2013) and in the case of TBI even up to 16 % (Annegers et al., 1996). The probability increases with the severity of the injury and is much higher in patients with late (more than 2 weeks) post-stroke (65-90%) or post-traumatic seizures (up to 86%), compared to patients with early seizures which usually develop within 24 hours (17-35%) (Haltiner AM, Temkin NR, Dikmen SS,Camilo & Goldstein, 2004). Therefore, according to the definition of the International League Against Epilepsy (ILAE) one late seizure per se can be diagnosed as epileptic disease (Jungehulsing et al., 2013) because the condition of an “enduring alteration of the brain that increases the likelihood of future seizures” (Fisher et al., 2005) is provided. Identifying the patients with acute brain injury who are prone to develop late seizures is a major therapeutic concern. So far, the pathophysiology of the injury, the severity of the lesion and EEG, have only limited prediction value. The discovery and validation of reliable biomarkers is therefore necessary.

Lesions that are accompanied by prolonged periods of blood-brain barrier (BBB) disruption might facilitate epileptogenesis (Seiffert et al., 2004; Shlosberg et al., 2010; van Vliet et al., 2007). Under these conditions serum components enter the interstitial space and electrolyte levels are altered in a way that provokes hyperexcitability. For example, lowered calcium and increased potassium concentrations have proconvulsant effects. On the other hand, the serum protein albumin probably activates astrocytes associated with downregulation of Kir channels (Ivens et al., 2007; Tomkins et al., 2007), neuronal depolarization and reduced buffering of glutamate and potassium.

Another electrophysiological phenomenon, which is commonly observed in acute brain injury and can facilitate lesion progression is cortical spreading depolarization (CSD) (Dohmen et al., 2008; Dreier et al., 2006; Oliveira-Ferreira et al., 2010). CSD is characterized by a breakdown of the electrochemical gradients across the neuronal membranes, which leads to massive glutamate

release, prolonged rise of intracellular calcium and massive depolarization of neurons and glial cells (Dreier, 2011; Somjen, 2001). The sustained depolarization of the neurons beyond the threshold for action potential generation prevents the sodium channels from recovering from inactivation. Generation of organized network activity is therefore blocked as is clinically evident in migraine aura (Hadjikhani et al., 2001). However, prolonged CSDs enhance hyperexcitability in the long run, probably due to excitatory plasticity processes (Berger et al., 2008; Ghadiri et al., 2012). I therefore became interested whether a causal relation exists between BBB disruption and CSD generation as a potential biomarker for development of chronic epilepsy. Furthermore, because prolonged seizures can cause rise in potassium and focal hypoxia, two potent agents provoking CSD, I tested whether CSDs co-occur with seizures in acutely compromised as well as in chronic epileptic tissue and can contribute to disease progression or even trigger a seizure.

Finally, another crucial question is how a seizure is triggered in the chronically epileptic brain. An epileptic seizure is defined by the ILAE as ”a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain”(Fisher et al., 2005) and is therefore likely to be triggered by a transient change in the brain state (Quilichini and Bernard, 2012). Such transitions in brain states are typically the switch from wakefulness to slow wave sleep and REM sleep. On electrophysiological level this would be the switch between theta and gamma oscillations which occur in the hippocampus during exploratory behavior and REM sleep and fast sharp wave ripple oscillations, which can be detected during rest and slow wave sleep and are implemented in memory consolidation (Buzsáki, 1996; Buzsáki et al., 1992). These transitions are believed to be at least partially controlled by acetylcholine, as it can induce gamma and theta oscillations (Fano et al., 2011; Teitelbaum et al., 1975) and can block sharp-wave ripples (Norimoto et al., 2012; Behrens et al., in preparation). Acetylcholine levels are high during exploratory behavior, during REM-sleep (Jasper and Tessier, 1971) and upon arousal (Klinkenberg et al., 2011). Indeed cholinergic activation can induce seizure-like events in the chronically epileptic entorhinal cortex (Zimmerman et al., 2008). I therefore became interested in the mechanisms involved in cholinergic ictogenesis.

I compared generation of seizures and CSDs in naïve rat tissue, in acutely injured brain as a result of photothrombotic stroke, as well as in chronic epileptic tissue from pilocarpine-treated rats and resectates from drug-resistant epileptic patients. The slices were examined electrophysiologically as to generation of spontaneous seizure-like events and CSDs, as well as to pharmacologically-induced epileptiform activity by cholinergic agonists, including blocker of the Kv7 blockers, which imitate the effect of acetylcholine on the muscarinic M1 receptor. The comparison was further complemented with histochemical analysis to study tissue morphology and changes in expression of Kv7 channels.

4. Materials and Methods

Animal procedures were performed in accordance with the guidelines of the European Communities Council and approved by the regional authority (LaGeSO Berlin). Male wistar rats were used for all animal experiments.

Induction of acute brain injury by photothrombotic stroke

The Rose bengal method was used as described previously (Stoll et al., 2009). Briefly, anesthetized rats (ketamine-xylazin 1.6 and 0.6 mg/kg body weight, respectively) received Rose bengal intravenously via a tail vein catheter) (20 mg/kg body weight). For induction of photothrombosis, the calvarium was exposed for 15 min to a halogen light beam that was vertically centered 1 mm posterior and 1 mm lateral from bregma using a stereotactic frame. Sham operated animals received either the light beam or Rose bengal alone (total 9 animals, 4 and 5 animals, respectively).

Pilocarpine treatment

As previously described ((Wozny et al., 2005), status epilepticus (SE) was induced via an intraperitoneal (i.p.) injection of the muscarinic agonist pilocarpine (340 mg/kg). In order to prevent peripheral side effects, the rats were pretreated with the muscarinic antagonist methyl-scopolamine (1 mg/kg) subcutaneously. The sham control group received 0.9% NaCl i.p. instead of pilocarpine. SE was terminated after 90 min by diazepam (i.p. 10 mg/kg). Epileptogenesis was confirmed two to three months later by video observation and registration of at least 3 seizures of Racine's scale stages

Slice preparation

Rats were decapitated under deep isoflurane anesthesia and the rapidly removed brain was transferred into ice-cold artificial cerebrospinal fluid (aCSF), equilibrated with 95% O_2–5% CO_2, containing in mM: NaCl 129, $NaHCO_3$ 21, KCl 3, $CaCl_2$ 1.6, $MgSO_4$ 1.8, NaH_2PO_4 1.25, and glucose 10. Horizontal hippocampal-entorhinal cortex slices, 400 µm thick, were prepared on a vibrating blade microtome (Leica Microsystems, Wetzlar, Germany) and immediately transferred to an interface chamber, perfused with carbogenated aCSF at 36 ± 0.5 °C (flow rate: ~1.8 ml/min, pH 7.4, osmolarity: 300 ± 3 mosmol/kg). Slices were left to recover for at least 2 h before recordings were started.

Recordings were performed in the presence of carbogenated albumin-free serum electrolyte solution. In some experiment, slices were perfused with a modified solution – albumin-free serum (afSERUM), containing in mM: 129 NaCl, 21 $NaHCO_3$, 1.25 NaH_2PO_4, 0.8 $MgSO_4$, 1.3 $CaCl_2$, 5.7 KCl, 10 glucose, 1 glutamine.

Human Neocortical Slices

The experiments were approved by the local ethics committee and written informed consent was obtained from each patient. Experiments were performed on neocortical tissue of patients suffering from drug-resistant temporal or frontal lobe epilepsy. In selected patients, subdural ECoG recordings were clinically indicated prior to surgery to locate the epileptic focus. Temporal or frontal lobe resectates from patients with intractable epilepsy were collected in the operating theater, and immediately immersed in ice-cold (4°C) carbogenated transport solution containing (in mM): 3 KCl , 1.25 NaH_2PO_4, 10 glucose, 2 $MgSO_4$, 2 $MgCl_2$, 1.6 $CaCl_2$, 21 $NaHCO_3$, 200 sucrose, 0.1 tocopherol, pH 7.4, osmolality 302 mOsm/kg. Neocortical slices (500µm) were obtained, transferred to a recording chamber and perfused with aCSF as described above. Recovery time of at least 5h was allowed before the recordings started.

Recording and Stimulation Electrodes

Field-potential recordings from the entorhinal and temporal cortex were performed with glass microelectrodes filled with aCSF. For additional registration of extracellular potassium fluctuations, double-barreled K^+-selective microelectrodes (Fluka 60031 ionophore, 150 mM NaCl), were prepared and tested as described previously (Petzold et al., 2005). Only electrodes showing peaks of 55-60 mV for a 10-fold change in potassium concentration were used. Bipolar stimulation electrodes were made of platinum wires, 50µm diameter, and 200µm tip separation. Single or paired stimuli (0.1 ms, 1-10 V, 50 ms interval) were delivered in the deep layers of the temporal and entorhinal cortex using a stimulus isolator in constant voltage mode (ISO Flex, AMPI Instruments, Jerusalem, Israel) controlled by a Master-8 (AMPI Instruments, Jerusalem, Israel).

Intrinsic Optical Imaging

The massive ion transfer during SD is accompanied by swelling of neurons, glial cells and cell organelles and can be optically detected as increase in the light scattering properties of the tissue. We monitored intrinsic optical signals with a CCD camera while transilluminating slices with white light. The signal was digitized with a frame grabber board. The first image in a series, captured before SD onset, served as control (T0) and was subtracted from each subsequent image, revealing changes in light transmittance (ΔT) over time. The image series was used to estimate the origin and spreading pattern of SD as well as to calculate its propagation velocity.

Histology

For morphological evaluation, Nissl staining and immunohistochemistry (IHC) were performed as described previously (Zahn et al., 2008). In short, deeply anesthetized sham control and treated rats (ketamine and xylazine,i.p., 7.2 mg and 1.12 mg/100 g of body weight, respectively) were transcardially perfused for 25 min with PGPic (4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4). The perfusion was initiated with plasma substitute (Deltadex 60, DeltaSelect, Pfullingen, Germany) for 10 s at 38 °C in order to prevent coagulation and the fixation was terminated with 5% sucrose in 0.1 M PB for 5 min in order ot remove excessive fixative. The brains were then rapidly removed, cryoprotected in 0.8 M sucrose and 0.1 M PB overnight, frozen in hexane at −60 °C and stored at −80 °C until processing.

For IHC, the diaminobenzidine (DAB) method was used (Brinschwitz et al., 2010). Horizontal freely floating sections (25 µm thick) obtained at a cryostat, were pretreated according to a standard protocol for background reduction and incubated with the primary antibodies (rabbit anti-KCNQ2 and anti- phosphoKCNQ3, Abcam, Cambridge, UK) for 48 h at 4 °C. Thereafter, sections were incubated for 24 h at 4 °C with the secondary antibody (biotinylated goat-anti-rabbit IgG, 1:2000; Vector Laboratories, Burlingame, USA). For signal amplification, the sections were treated for 12 h with avidin–biotin–peroxidase complex solution (1:200 in PBS, Vector Laboratories) and peroxidase activity was revealed by incubation in 0.05% DAB, 10 mM imidazole and 0.0015% hydrogen peroxide in 50 mM Tris buffer, pH 7.6 for 15min.

For immunofluorescence staining, 25 µm thick sections were incubated with rabbit anti-NeuN (1:500, Sigma-Aldrich, Germany). Signal detection was achieved by incubation with Alexa Fluor 568 goat anti-rabbit secondary antibody (1:200, MoBiTec, Göttingen, Germany) for two h at room temperature

Data analysis

Electrophysiological data obtained with Spike2 software were exported to Matlab and analyzed using “homemade” scripts. For analysis of the epileptiform activity, data were low pass filtered at 3 Hz and the amplitude was defined as the maximum peak to peak fluctuation. Power spectrum analysis was performed with Spike2 software. For statistical analysis, Wilcoxon rank-sum test, paired t-test, and one-way Anova were used.

For analysis of potassium channel expression levels, photomicrographs of the entorhinal cortex were obtained with a digital camera (HCS MXR Vision Technology, The Netherlands) on a Leica microscope with a 10x magnification objective and constant illumination and exposure settings. Altogether 4 sections from control rats and 7 sections from pilocarpine-treated rats stained with KCNQ2 were

evaluated. For KCNQ3, the number of sections was 6 and 9 from the control and epileptic group respectively. From each photograph, a band with the same width (10 pixels) and length from alveus to layer I of the medial EC was cropped and exported to Matlab. The signal intensity was measured on a scale from 0 to 100 and averaged for each layer of the EC. One-way ANOVA was used to compare the intensity within layers in control and pilocarpine-treated epileptic animals.

5. Results

Acutely compromised tissue is prone to seizure and CSD generation

Photothrombotic stroke was used as a model of acute brain injury. Induction of a focal neocortical infarct through the intact calvarium was associated with a large-scale disruption of the blood brain barrier, also in the hippocampus. Therefore, field potentials were recorded from acute hippocampal slices at 24 h or 7 days post induction of stroke. In slices perfused with standard artificial cerebrospinal fluid (aCSF, see methods) spontaneous pathologic activity was missing. However CSDs could be easily induced by recurrent repetitive electrical stimulation in 7 slices (78%), suggesting a lower threshold for induction of CSDs in the peri-ischemic tissue, compared to sham operated animals. Acute brain injury is often accompanied by disruption of the blood-brain-barrier (BBB), which allows ions to invade the interstitial space. We mimicked the disturbed extracellular electrolyte environment during BBB dysfunction by replacing the aCSF with a modified solution, resembling serum concentrations (afSERUM, see methods). Under these conditions, slices recorded 24 h after photothrombosis, presented a high rate of spontaneous CSDs sometimes accompanied by seizure-like events (n = 8 /10 slices). In 6 slices, fast epileptiform activity preceded CSDs. By contrast, under the same conditions (afSERUM) , spontaneous CSDs never emerged in slices from naïve animals (n = 16) or in slices obtained 7 days after photothrombosis (n = 9), at a time when vascular permeability had returned to control levels. It seems therefore that altered electrolyte concentrations are necessary, but are not the sole reason for generation of abnormal activity.

Serum proteins also enter the interstitial space during BBB disruption and might contribute to seizure generation (Ivens et al., 2007). To simulate albumin extravasation, we injected bovine-serum-albumin (BSA) into the right lateral ventricle to allow its diffusion into the adjacent hippocampus. In slices from albumin-injected animals, CSDs and seizures did not occur spontaneously. However they could be induced by electrical stimulation in 10% of slices perfused with aCSF and even in 80% of slices perfused with afSERUM.

The injected albumin was conjugated to fluorescein isothiocyanate conjugate (FITC), which enabled later detection. Immunostaining of slices 24 h post in-vivo injection of FITC-albumin showed

extracellular and intracellular staining in both hippocampi. Consistent with previous data from the neocortex (Ivens et al., 2007), the uptake of albumin was mostly into NeuN-negative non-neuronal glial cells (Fig. 2A2 from (Lapilover et al., 2012)).

Chronic epileptic tissue has an increased threshold for cortical spreading depolariztion

To determine resistance properties of chronically epileptic tissue, I examined the susceptibility to CSD induced by aCSF containing increasing concentrations of potassium ($[K^+]_o$, in mM: 17.5 - 25, in steps of 2.5 every 30 min) in chronically epileptic human neocortex, chronically epileptic pilocarpine-treated rats and sham-control rats. The potassium concentration required for CSD induction was significantly lower in the control rats compared to the pilocarpine-treated chronically epileptic rats and the human slices, whereas the threshold was similar in the two epileptic groups. Linear regression of the pooled data of all groups demonstrated that the time until the first CSD correlated significantly with $[K^+]_o$ as measured in the tissue with ion-sensitive electrodes immediately before the first CSD ($R = 0.508$, $p = 0.004$). This indicates a difference in tissue tolerance toward higher levels of $[K^+]_o$, rather than different rates of potassium buffering among the groups. These results were confirmed in a different experimental paradigm where slices from all groups were exposed to the same concentration of $[K^+]_o$ (25 mM, 45 min). Under these conditions, CSDs occurred in all slices. However, the CSD incidence was significantly higher in the sham animals (8.3 ± 4.1 CSDs, n = 8), compared to the other 2 groups which had similar incidences (4.0 ± 2.1 CSDs, n = 6 and 2.0 ± 1.5 CSDs, n = 6 respectively). Under both experimental conditions, the DC shifts in the epileptic rats (9.0 ± 3.2 mV) were significantly smaller compared to the control rats (14.5 ± 2.7 mV), possibly due to cell loss in the epileptic tissue, resulting in a lower number of cells recruited.

Chronic epileptic tissue demonstrates altered sensitivity to bicuculline

GABA-ergic signaling is altered in chronic epilepsy. While loss of pyramidal cells leads to disinhibition by increased reconnection of interneurons on the remaining interneurons (Morin et al., 1998), increased perisomatic sprouting of interneuronal axons on granule cells might enhance synchronization (Maglóczky and Freund, 2005). Furthermore, a role for depolarizing GABA has been proposed (Cohen et al., 2002). I therefore compared the effect of blocking GABA-ergic signaling on the induction of CSDs and epileptic seizures in the naive and chronically epileptic tissue. Consistent with previous studies, bicuculline (50 µM) readily induced epileptiform activity in control rats (7 out of 9 slices). In 4 slices bicuculline additionally induced CSDs. By contrast, neither epileptiform activity nor CSDs could be detected with bicuculline in the epileptic rat and human slices. Interestingly, when

co-applied with 25 mM K^+, 50 µM bicuculline significantly increased the incidence of CSDs in all tested groups: sham controls (8.3 ± 4.1 versus 13.4 ± 4.3 CSDs, n = 8, t test, p = 0.03), pilocarpine-treated rats (4.0 ± 2.1 versus 7.7 ± 2.0 CSDs, n = 6, p = 0.011) and human epileptic slices (2.0 ± 1.5 versus 4.4 ± 0.9 CSDs, n = 5, p = 0.014). These results indicate that the mechanisms of induction of epileptiform discharges, as well as of CSD, differ between healthy and chronically reorganized cortex.

Chronic epileptic tissue demonstrates altered sensitivity to acetylcholine

Acetylcholine (ACh) is involved in the regulation of wakefulness and attention, which are associated in-vivo with gamma and theta oscillations(Buzsaki, 2006; Klinkenberg et al., 2011; Teitelbaum et al., 1975). These can be induced by ACh also in the hippocampal slice preparation (Decker et al., 2009; Fano et al., 2011). However, in epileptic tissue ACh induces readily epileptiform activity (Zimmerman et al., 2008). I compared activity induced by increasing concentrations of ACh in the entorhinal cortex of control and pilocarpine-treated rats (Fig. 1 from (Maslarova et al., 2013)). In slices from control animals, high concentrations of ACh induced short recurrent discharges in the gamma frequency range, nested on a slow wave. By contrast, in the pilocarpine-treated rats, 5 µM ACh were sufficient for induction of short recurrent epileptiform discharges in all slices (n= 11) and 20 µM, ACh additionally induced longer but less frequent epileptiform events in 7 out of 11 slices).

Epileptogenic effect of the Kv7 blocker Linopirdine

Cholinergic epileptiform activity in kainate- and pilocarpine-treated rats is sensitive to atropine, suggesting involvement of muscarinic receptors. However, changes in expression of muscarinic receptors do not seem to be involved (Zimmerman et al., 2008). I therefore hypothesized that the increased sensitivity to ACh in epileptic tissue might be due to alterations of muscarinic receptor-associated ion channels. Voltage-gated Kv7 potassium channels were a likely candidate, because they mediate a slowly activating depolarization-induced outward current and help to maintain the resting membrane potential and to prevent prolonged bursting (Brown and Adams, 1980; Yue and Yaari, 2004). This so-called M-current received its name because it is blocked by activation of muscarinic M1 receptors. Mutations of Kv7.2 and Kv7.3 subunits are associated with some infantile epilepsies (Biervert, 1998; Schroeder et al., 1998). I compared spontaneous activity in entorhinal cortex from epileptic rats in response to ACh and the M-current blocker linopirdine. In control slices, linopirdine had no effect on baseline activity, with one exception, where 20 µM linopirdine induced short tiny epileptiform discharges (0.56 s duration, 0.12 mV amplitude, recurrence rate of 10.4/ min) which increased in duration (0.67 s) and incidence (15.3/ min) when the concentration was increased to 50

µM. However, in epileptic tissue, 20 µM linopirdine induced epileptiform activity in the majority of slices (n = 11/ 12) (Fig. 2A from Maslarova et al., 2013).

The Kv7 opener retigabine completely abolished epileptiform activity induced by linopirdine in 5 slices and reduced their incidence in one additional slice (Fig. 3A and B from Maslarova et al., 2013), confirming that the ictogenic effect of linopirdine was due to its action on Kv7 channels, specifically Kv7.2/ 3 or Kv7.5 as the Kv7.1 opener L364-373 had only a slight modulating effect on the overall area under the curve of the trace.

Expression of voltage-gated Kv7 potassium channels is altered in chronically epileptic tissue

Next, I compared the expression of Kv7.2 and Kv7.3 subunits in control and epileptic tissue by immuncytochemistry. A quantification of these results is shown in Figures 5 and 6 from (Maslarova et al., 2011). Under control conditions, KCNQ2 was expressed perisomatically in all layers of the entorhinal cortex and especially in layer III appearing as a darker band (n = 4 sections from 2 animals). This staining pattern of layer III was missing in the epileptic brains (n = 7 sections from 3 animals), probably due to the well-described massive neuronal death in EC layer III (Du et al., 1995). However, perisomatic staining was reduced in other layers as well compared to control.

KCNQ3, known to form heteromers with KCNQ2, was expressed in control animals in apical dendrites of layer I/II and partially in some cell bodies in layer III of the EC (n = 6 sections from 2 animals). In the pilocarpine animals, the KCNQ3 staining was reduced in layer I/II of the EC (n = 9 sections from 3 animals). In the subiculum, marked expression of KCNQ3 was present in dendrites extending through all subicular layers in control animals. In pilocarpine-treated animals, the number of KCNQ3-positive dendrites was strongly reduced and the KCNQ3-positive segments were shorter.

6. Discussion

In my MD/PhD thesis, I have studied the sensitivity to induction of CSD and epileptic seizures in healthy brain, in models of acute brain injury (photothrombotic stroke and albumin exposure) and in chronically epileptic tissue obtained from an animal model or from patients with temporal lobe epilepsy (TLE).

Similarly to clinical data, acute injury by photothrombotic stroke increased susceptibility in *ex-vivo* brain slices to generation of CSD and epileptiform activity. This required perfusion with aCSF, modified to resemble serum electrolyte composition, as in BBB-disruption. Application of albumin to healthy tissue partially mimicked the impact of photothrombotic stroke, implying that BBB disruption and albumin extravasation can cause the pathologic activity after acute injury. Albumin in the interstitial space is predominantly taken up by astrocytes, thus probably leading to their activation and to impairment of potassium buffering (Ivens et al., 2007; Tomkins et al., 2007). Accumulated extracellular potassium increases tissue excitability (Somjen, 2002). Combined with an additional stimulus, for example in the form of energy compromise in stroke or mechanic stimulation in trauma, this hyperexcitable state can be easily transformed into a seizure or a CSD. It is therefore crucial to control BBB-disruption in acute injury.

By contrast, chronically epileptic tissue showed increased resistance to CSD induction and often also to seizure induction by bicuculline. Similar resistance of the epileptic tissue has been described for other convulsants, such as 4-AP (Zahn et al., 2008). Increased threshold for CSD generation has been described before for the kindling model (Koroleva et al., 1993) and after BBB-disruption (Seiffert et al., 2004; Tomkins et al., 2007). The increased threshold therefore seems to be specific to the epileptic tissue rather than to the initial stimulus. Because the potassium concentration measured in the tissue on CSD onset varied between the groups, the increased resistance in the epileptic tissue was probably due to a higher tolerance to potassium. Blocking GABA-ergic signaling with bicuculline reduced the threshold for CSD in the healthy brain similarly to the epileptic tissue. Therefore reorganization of inhibitory connections, as is described for chronically epileptic tissue, is unlikely the reason for the increased resistance to CSD generation.

Reorganization of GABA-ergic connections in the epileptic hippocampus however might play a role for the switch in the nature of epileptogenic stimuli. Thus, in the epileptic brain, GABA-mediated responses can be excitatory (Huberfeld et al., 2008, 2007) and blockade of GABA-ergic transmission will therefore exert a different effect from control tissue. In fact, in human cortex and hippocampus from TLE patients, most methods for induction of seizure-like events, such as lowering of the Ca^{2+} or Mg^{2+} concentration, fail. Potassium can still induce epileptiform activity in the hippocampus, however at concentrations close to ceiling levels, that are otherwise only reached during activity-

dependent rises in potassium concentration (Gabriel et al., 2004). In neocortical tissue, elevation of potassium does not induce seizure-like events unless combined with another convulsant. This indicates that homeostatic mechanisms are activated during epileptogenesis which try to keep neuronal activity within physiological limits. At present there are only accidental observations in this respect. Upregulation of opioid receptors in the amygdala upon kindling (Rocha et al., 1996, 1994) has been proposed to have a protective function by limiting seizure propagation. Similarly, transient expression of GAD in granule cell upon repetitive stimulation leads to consequent corelease of GABA and glutamate from mossy fiber terminals, thus silencing CA3 neurons (Gutiérrez and Heinemann, 2001). More recently it was shown that there is activity dependent editing of Kv1 channels which could account for lowered sensitivity to 4-AP (Zahn et al., 2008) and activity-dependent editing of the alpha 3 subunit of glycine receptors with increased affinity to glycine and some of its agonists (Meier et al., 2005). It may be useful to direct new research strategies onto the discovery of inherent antiepileptic mechanisms. However this does not apply to all potential proconvulsive mechanisms.

Homeostatic plasticity in the compromised brain might become pathogenic and thus contribute to epileptogenesis. The excitatory transmitter and neuromodulator acetylcholine, which is implicated in learning and regulation of attention can induce theta and superimposed gamma oscillations in the healthy brain, demonstrated strong ictogenic properties in chronically epileptic tissue. Acetylcholine levels vary in the hippocampus depending on the attentional state and thus are important in the switch between wakefulness and exploratory behavior on the one side which are associated with high acetylcholine levels and sleep and memory consolidation on the other side, when acetylcholine concentrations are low. Therefore the ictogenic effect of this neurotransmitter is of interest especially for epilepsies upon arousal (Novakova et al., 2013).

ACh can block Kv7 potassium channels by its action on muscarinic M1 receptors (Brown and Adams, 1980). In our experiments, a selective blocker of Kv7 channels also induced epileptiform activity in epileptic rats even though it failed to do so in the control rats. It is therefore likely that changes in the expression of Kv7 channels are involved in the acquired convulsive properties of ACh. Accordingly, we observed reduced expression of Kv7.2 and Kv7.3 in the entorhinal cortex and subiculum from pilocarpine-treated animals. Loss of function mutations of these channels have been described in several genetic symptoms associated with overexcitability of the tissue such as neuropathic pain (Maljevic et al., 2008) and importantly benign familial neonatal convulsions (Biervert, 1998). The KCNQ1 channel, which until recently was only described in the heart as the carrier of long QT syndrome, seems to be expressed also in the brain and associated with SUDEP (sudden unexpected death in epilepsy) (Goldman et al., 2009).

The reduced expression of Kv7 channels in the chronic epileptic entorhinal cortex might have several reasons. On the one hand, underexpression might be due to atrophy of the Kv7.2/7.3 positive neurons. Excessive cell death in layer III of the entorhinal cortex is well described in models of temporal lobe epilepsy (Du et al., 1993; Tolner et al., 2007) and was also observed in my experiments. In addition, channels can be destroyed by the formation of auto-antibodies. This has been described for voltage gated potassium channels after limbic encephalitis as well as for glutamate receptors (Irani and Vincent, 2011; Kröll-Seger et al., 2009). Recent evidence points at formation of autoantibodies also in chronic temporal lobe epilepsy (Vezzani et al., 2011; Vincent and Bien, 2008). Underexpression of Kv7 channel subunits alone cannot explain the ictogenic effect of the Kv7 blocker, as becomes clear from our control experiment, where linopirdine did not induce epileptiform activity. More likely, Kv7 channels in the epileptic brain become crucial in compensating hyperexcitability, caused by other factors. Thus, Kv7 channels can control cell bursting induced by the persistent sodium current, which is overexpressed in epileptic CA1 (Chen et al., 2011; Yue and Yaari, 2004).

In conclusion, hyperexcitability in healthy and chronically epileptic tissue is driven by different mechanisms and cannot be compared. While in the acutely compromised brain, BBB-disruption facilitates pathologic activity via altered electrolyte concentrations and impaired potassium buffering by the astrocytes due to albumin, in the chonic epileptic tissue, changes in the homeostatic plasticity take place which try to compensate for the induction of seizures. However these changes in expression and function of ion channels and neurotransmitters seem, as a side effect, to convey totally new types of hyperexcitability. These differences should be considered when planning therapeutic strategies for epilepsy treatment.

7. References

Annegers, J.F., Rocca, W.A., Hauser, W.A., 1996. Causes of epilepsy: contributions of the Rochester epidemiology project. Mayo Clinic proceedings. Mayo Clinic 71, 570–5.

Berger, M., Speckmann, E.-J., Pape, H.C., Gorji, A., 2008. Spreading depression enhances human neocortical excitability in vitro. Cephalalgia : an international journal of headache 28, 558–62.

Bernard, C., Anderson, A., Becker, A., Poolos, N.P., Beck, H., Johnston, D., 2004. Acquired dendritic channelopathy in temporal lobe epilepsy. Science (New York, N.Y.) 305, 532–5.

Biervert, C., 1998. A Potassium Channel Mutation in Neonatal Human Epilepsy. Science 279, 403–406.

Bolkvadze, T., Pitkänen, A., 2012. Development of post-traumatic epilepsy after controlled cortical impact and lateral fluid-percussion-induced brain injury in the mouse. Journal of neurotrauma 29, 789–812.

Brinschwitz, K., Dittgen, A., Madai, V.I., Lommel, R., Geisler, S., Veh, R.W., 2010. Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain. Neuroscience 168, 463–76.

Brown, D.A., Adams, P.R., 1980. Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. Nature 283, 673–6.

Buzsaki, G., 2006. Rhythms of the Brain. Oxford University Press, USA.

Buzsáki, G., 1996. The Hippocampo-Neocortical Dialogue. Cerebral Cortex 6, 81–92.

Buzsáki, G., Horváth, Z., Urioste, R., Hetke, J., Wise, K., 1992. High-frequency network oscillation in the hippocampus. Science (New York, N.Y.) 256, 1025–7.

Camilo, O., Goldstein, L.B., 2004. Seizures and epilepsy after ischemic stroke. Stroke; a journal of cerebral circulation 35, 1769–75.

Chen, S., Su, H., Yue, C., Remy, S., Royeck, M., Sochivko, D., Opitz, T., Beck, H., Yaari, Y., 2011. An increase in persistent sodium current contributes to intrinsic neuronal bursting after status epilepticus. Journal of neurophysiology 105, 117–29.

Cohen, I., Navarro, V., Clemenceau, S., Baulac, M., Miles, R., 2002. On the origin of interictal activity in human temporal lobe epilepsy in vitro. Science (New York, N.Y.) 298, 1418–21.

Decker, J.M., Wójtowicz, A.M., Ul Haq, R., Braunewell, K.-H., Heinemann, U., Behrens, C.J., 2009. C-type natriuretic peptide decreases hippocampal network oscillations in adult rats in vitro. Neuroscience 164, 1764–75.

Dohmen, C., Sakowitz, O.W., Fabricius, M., Bosche, B., Reithmeier, T., Ernestus, R.-I., Brinker, G., Dreier, J.P., Woitzik, J., Strong, A.J., Graf, R., 2008. Spreading depolarizations occur in human ischemic stroke with high incidence. Annals of neurology 63, 720–8.

Dreier, J.P., 2011. The role of spreading depression, spreading depolarization and spreading ischemia in neurological disease. Nature medicine 17, 439–47.

Dreier, J.P., Major, S., Pannek, H.-W., Woitzik, J., Scheel, M., Wiesenthal, D., Martus, P., Winkler, M.K.L., Hartings, J.A., Fabricius, M., Speckmann, E.-J., Gorji, A., 2012. Spreading convulsions, spreading depolarization and epileptogenesis in human cerebral cortex. Brain : a journal of neurology 135, 259–75.

Dreier, J.P., Woitzik, J., Fabricius, M., Bhatia, R., Major, S., Drenckhahn, C., Lehmann, T.-N., Sarrafzadeh, A., Willumsen, L., Hartings, J.A., Sakowitz, O.W., Seemann, J.H., Thieme, A., Lauritzen, M., Strong, A.J., 2006. Delayed ischaemic neurological deficits after subarachnoid haemorrhage are associated with clusters of spreading depolarizations. Brain : a journal of neurology 129, 3224–37.

Du, F., Eid, T., Lothman, E.W., Köhler, C., Schwarcz, R., 1995. Preferential neuronal loss in layer III of the medial entorhinal cortex in rat models of temporal lobe epilepsy. The Journal of neuroscience : the official journal of the Society for Neuroscience 15, 6301–13.

Du, F., Whetsell, W.O., Abou-Khalil, B., Blumenkopf, B., Lothman, E.W., Schwarcz, R., 1993. Preferential neuronal loss in layer III of the entorhinal cortex in patients with temporal lobe epilepsy. Epilepsy research 16, 223–33.

Dubé, C.M., Brewster, A.L., Richichi, C., Zha, Q., Baram, T.Z., 2007. Fever, febrile seizures and epilepsy. Trends in neurosciences 30, 490–6.

Ellerkmann, R.K., Remy, S., Chen, J., Sochivko, D., Elger, C.E., Urban, B.W., Becker, A., Beck, H., 2003. Molecular and functional changes in voltage-dependent Na(+) channels following pilocarpine-induced status epilepticus in rat dentate granule cells. Neuroscience 119, 323–33.

Fano, S., Caliskan, G., Behrens, C.J., Heinemann, U., 2011. Histaminergic modulation of acetylcholine-induced γ oscillations in rat hippocampus. Neuroreport 22, 520–4.

Ferando, I., Mody, I., 2012. GABAA receptor modulation by neurosteroids in models of temporal lobe epilepsies. Epilepsia 53 Suppl 9, 89–101.

Fisher, R.S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., Engel, J., 2005. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46, 470–2.

Friedman, A., Behrens, C.J., Heinemann, U., 2007. Cholinergic dysfunction in temporal lobe epilepsy. Epilepsia 48 Suppl 5, 126–30.

Gabriel, S., Njunting, M., Pomper, J.K., Merschhemke, M., Sanabria, E.R.G., Eilers, A., Kivi, A., Zeller, M., Meencke, H.-J., Cavalheiro, E.A., Heinemann, U., Lehmann, T.-N., 2004. Stimulus and potassium-induced epileptiform activity in the human dentate gyrus from patients with and without hippocampal sclerosis. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 10416–30.

Ghadiri, M.K., Kozian, M., Ghaffarian, N., Stummer, W., Kazemi, H., Speckmann, E.-J., Gorji, A., 2012. Sequential changes in neuronal activity in single neocortical neurons after spreading depression. Cephalalgia : an international journal of headache 32, 116–24.

Goldman, A.M., Glasscock, E., Yoo, J., Chen, T.T., Klassen, T.L., Noebels, J.L., 2009. Arrhythmia in heart and brain: KCNQ1 mutations link epilepsy and sudden unexplained death. Science translational medicine 1, 2ra6.

Gutiérrez, R., Heinemann, U., 2001. Kindling induces transient fast inhibition in the dentate gyrus--CA3 projection. The European journal of neuroscience 13, 1371–9.

Hadjikhani, N., Sanchez Del Rio, M., Wu, O., Schwartz, D., Bakker, D., Fischl, B., Kwong, K.K., Cutrer, F.M., Rosen, B.R., Tootell, R.B., Sorensen, A.G., Moskowitz, M.A., 2001. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. Proceedings of the National Academy of Sciences of the United States of America 98, 4687–92.

Huberfeld, G., Clemenceau, S., Cohen, I., Pallud, J., Wittner, L., Navarro, V., Baulac, M., Miles, R., 2008. [Epileptiform activities generated in vitro by human temporal lobe tissue]. Neuro-Chirurgie 54, 148–58.

Huberfeld, G., Wittner, L., Clemenceau, S., Baulac, M., Kaila, K., Miles, R., Rivera, C., 2007. Perturbed chloride homeostasis and GABAergic signaling in human temporal lobe epilepsy. The Journal of neuroscience : the official journal of the Society for Neuroscience 27, 9866–73.

Irani, S.R., Vincent, A., 2011. NMDA receptor antibody encephalitis. Current neurology and neuroscience reports 11, 298–304.

Ivens, S., Kaufer, D., Flores, L.P., Bechmann, I., Zumsteg, D., Tomkins, O., Seiffert, E., Heinemann, U., Friedman, A., 2007. TGF-beta receptor-mediated albumin uptake into astrocytes is involved in neocortical epileptogenesis. Brain : a journal of neurology 130, 535–47.

Jasper, H.H., Tessier, J., 1971. Acetylcholine liberation from cerebral cortex during paradoxical (REM) sleep. Science (New York, N.Y.) 172, 601–2.

Jungehulsing, G.J., Heuschmann, P.U., Holtkamp, M., Schwab, S., Kolominsky-Rabas, P.L., 2013. Incidence and predictors of post-stroke epilepsy. Acta neurologica Scandinavica 127, 427–30.

Klinkenberg, I., Sambeth, A., Blokland, A., 2011. Acetylcholine and attention. Behavioural brain research 221, 430–42.

Koroleva, V.I., Vinogradova, L. V, Bures, J., 1993. Reduced incidence of cortical spreading depression in the course of pentylenetetrazol kindling in rats. Brain research 608, 107–14.

Kröll-Seger, J., Bien, C.G., Huppertz, H.-J., 2009. Non-paraneoplastic limbic encephalitis associated with antibodies to potassium channels leading to bilateral hippocampal sclerosis in a pre-pubertal girl. Epileptic disorders : international epilepsy journal with videotape 11, 54–9.

Lapilover, E.G., Lippmann, K., Salar, S., Maslarova, A., Dreier, J.P., Heinemann, U., Friedman, A., 2012. Peri-infarct blood-brain barrier dysfunction facilitates induction of spreading depolarization associated with epileptiform discharges. Neurobiology of disease 48, 495–506.

Maglóczky, Z., Freund, T.F., 2005. Impaired and repaired inhibitory circuits in the epileptic human hippocampus. Trends in neurosciences 28, 334–40.

Maljevic, S., Wuttke, T. V, Lerche, H., 2008. Nervous system KV7 disorders: breakdown of a subthreshold brake. The Journal of physiology 586, 1791–801.

Maslarova, A., Alam, M., Reiffurth, C., Lapilover, E., Gorji, A., Dreier, J.P., 2011. Chronically epileptic human and rat neocortex display a similar resistance against spreading depolarization in vitro. Stroke; a journal of cerebral circulation 42, 2917–22.

Maslarova, A., Salar, S., Lapilover, E., Friedman, A., Veh, R.W., Heinemann, U., 2013. Increased susceptibility to acetylcholine in the entorhinal cortex of pilocarpine-treated rats involves alterations in KCNQ channels. Neurobiology of disease.

McClelland, S., Dubé, C.M., Yang, J., Baram, T.Z., 2011. Epileptogenesis after prolonged febrile seizures: mechanisms, biomarkers and therapeutic opportunities. Neuroscience letters 497, 155–62.

Meier, J.C., Henneberger, C., Melnick, I., Racca, C., Harvey, R.J., Heinemann, U., Schmieden, V., Grantyn, R., 2005. RNA editing produces glycine receptor alpha3(P185L), resulting in high agonist potency. Nature neuroscience 8, 736–44.

Morin, F., Beaulieu, C., Lacaille, J.C., 1998. Cell-specific alterations in synaptic properties of hippocampal CA1 interneurons after kainate treatment. Journal of neurophysiology 80, 2836–47.

Norimoto, H., Mizunuma, M., Ishikawa, D., Matsuki, N., Ikegaya, Y., 2012. Muscarinic receptor activation disrupts hippocampal sharp wave-ripples. Brain research 1461, 1–9.

Novakova, B., Harris, P.R., Ponnusamy, A., Reuber, M., 2013. The role of stress as a trigger for epileptic seizures: A narrative review of evidence from human and animal studies. Epilepsia 54, 1866–76.

Oliveira-Ferreira, A.I., Milakara, D., Alam, M., Jorks, D., Major, S., Hartings, J.A., Lückl, J., Martus, P., Graf, R., Dohmen, C., Bohner, G., Woitzik, J., Dreier, J.P., 2010. Experimental and preliminary clinical evidence of an ischemic zone with prolonged negative DC shifts surrounded by a normally perfused tissue belt with persistent electrocorticographic depression. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 30, 1504–19.

Petzold, G.C., Windmüller, O., Haack, S., Major, S., Buchheim, K., Megow, D., Gabriel, S., Lehmann, T.-N., Drenckhahn, C., Peters, O., Meierkord, H., Heinemann, U., Dirnagl, U., Dreier, J.P., 2005. Increased extracellular K+ concentration reduces the efficacy of N-methyl-D-aspartate receptor antagonists to block spreading depression-like depolarizations and spreading ischemia. Stroke; a journal of cerebral circulation 36, 1270–7.

Quilichini, P.P., Bernard, C., 2012. Brain state-dependent neuronal computation. Frontiers in computational neuroscience 6, 77.

Rocha, L., Briones, M., Ackermann, R.F., Anton, B., Maidment, N.T., Evans, C.J., Engel, J., 1996. Pentylenetetrazol-induced kindling: early involvement of excitatory and inhibitory systems. Epilepsy research 26, 105–13.

Rocha, L., Maidment, N.T., Evans, C.J., Ackermann, R.F., Engel, J., 1994. Microdialysis reveals changes in extracellular opioid peptide levels in the amygdala induced by amygdaloid kindling stimulation. Experimental neurology 126, 277–83.

Schroeder, B.C., Kubisch, C., Stein, V., Jentsch, T.J., 1998. Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K+ channels causes epilepsy. Nature 396, 687–90.

Seiffert, E., Dreier, J.P., Ivens, S., Bechmann, I., Tomkins, O., Heinemann, U., Friedman, A., 2004. Lasting blood-brain barrier disruption induces epileptic focus in the rat somatosensory cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 7829–36.

Shlosberg, D., Benifla, M., Kaufer, D., Friedman, A., 2010. Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. Nature reviews. Neurology 6, 393–403.

Somjen, G.G., 2001. Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. Physiological reviews 81, 1065–96.

Somjen, G.G., 2002. Ion regulation in the brain: implications for pathophysiology. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 8, 254–67.

Stoll, G., Kleinschnitz, C., Meuth, S.G., Braeuninger, S., Ip, C.W., Wessig, C., Nölte, I., Bendszus, M., 2009. Transient widespread blood-brain barrier alterations after cerebral photothrombosis as revealed by gadofluorine M-enhanced magnetic resonance imaging. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 29, 331–41.

Teitelbaum, H., Lee, J.F., Johannessen, J.N., 1975. Behaviorally evoked hippocampal theta waves: a cholinergic response. Science (New York, N.Y.) 188, 1114–6.

Tolner, E.A., Frahm, C., Metzger, R., Gorter, J.A., Witte, O.W., Lopes da Silva, F.H., Heinemann, U., 2007. Synaptic responses in superficial layers of medial entorhinal cortex from rats with kainate-induced epilepsy. Neurobiology of disease 26, 419–38.

Tomkins, O., Friedman, O., Ivens, S., Reiffurth, C., Major, S., Dreier, J.P., Heinemann, U., Friedman, A., 2007. Blood-brain barrier disruption results in delayed functional and structural alterations in the rat neocortex. Neurobiology of disease 25, 367–77.

Van Vliet, E.A., da Costa Araújo, S., Redeker, S., van Schaik, R., Aronica, E., Gorter, J.A., 2007. Blood-brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain : a journal of neurology 130, 521–34.

Vezzani, A., French, J., Bartfai, T., Baram, T.Z., 2011. The role of inflammation in epilepsy. Nature reviews. Neurology 7, 31–40.

Vincent, A., Bien, C.G., 2008. Anti-NMDA-receptor encephalitis: a cause of psychiatric, seizure, and movement disorders in young adults. Lancet neurology 7, 1074–5.

Wozny, C., Knopp, A., Lehmann, T.-N., Heinemann, U., Behr, J., 2005. The subiculum: a potential site of ictogenesis in human temporal lobe epilepsy. Epilepsia 46 Suppl 5, 17–21.

Yue, C., Yaari, Y., 2004. KCNQ/M channels control spike afterdepolarization and burst generation in hippocampal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 4614–24.

Zahn, R.K., Tolner, E.A., Derst, C., Gruber, C., Veh, R.W., Heinemann, U., 2008. Reduced ictogenic potential of 4-aminopyridine in the perirhinal and entorhinal cortex of kainate-treated chronic epileptic rats. Neurobiology of disease 29, 186–200.

Zimmerman, G., Njunting, M., Ivens, S., Tolner, E.A., Tolner, E., Behrens, C.J., Gross, M., Soreq, H., Heinemann, U., Friedman, A., 2008. Acetylcholine-induced seizure-like activity and modified cholinergic gene expression in chronically epileptic rats. The European journal of neuroscience 27, 965–75.

8. Eidesstattliche Versicherung

„Ich, Anna Maslarova, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: **"Different susceptibility of acutely injured and chronic epileptic brain tissue to induction of cortical spreading depolarization and seizures"** selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)" des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

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Datum Unterschrift

9. Declaration of own contribution

The detailed contributions of the doctoral candidate Anna Maslarova to the submitted publications are listed:

• **Publication 1**

Maslarova A, Alam M, Reiffurth C, Lapilover E, Gorji A and Dreier JP

"Chronically Epileptic Human and Rat Neocortex Display a Similar Resistance Against Spreading Depolarization In Vitro"

Stroke. 2011;42:2917-2922

Contribution in percent: 70%;

Detailed contribution: Active participation in planning the study and all experiments; conduction and analysis of the experiments (extracellular recordings of field potential and potassium changes in horizontal temporo-hippocampal rat slices and human neocortical slices, intrinsic optical imaging); presentation of the results at international conferences and meetings (BNF 2008, FENS 2008); active participation in writing the manuscript.

• **Publication 2**

Lapilover EG, Lippmann K, Salar S, **Maslarova A**, Dreier JP, Heinemann U, Friedman A

„Peri-infarct blood–brain barrier dysfunction facilitates induction of spreading

depolarization associated with epileptiform discharges"

Neurobiology of Disease 48 (2012) 495–506

Contribution in percent: 10%;

Detailed contribution: Participation in planning certain experiments of the study .Conduction and analysis of key experiments (perfusion, fixation and immunohistochemical stainings, microscopy); Editing of the manuscript.

• **Publication 3**

Maslarova A, Salar S, Lapilover E, Friedman A, Veh RW, Heinemann U

"Increased susceptibility to acetylcholine in the entorhinal cortex of pilocarpine-treated rats involves alterations in KCNQ channels"

Neurobiology of Disease. 2013 Aug;56:14-24.

Contribution in percent: 70%;

Detailed contribution: Active participation in planning the study and all experiments; conduction and analysis of the experiments (extracellular field potential recordings in horizontal temporo-hippocampal rat slices, video-analysis of seizure occurrence in the pilocarpine-treated rats, perfusion, fixation and immunohistochemical stainings, microscopy), presentation of the results at international conferences and meetings (Annual Epicure Conference Marseille 2010, Society for Neuroscience 2010; Berlin Brain Days 2010); active participation in writing the manuscript; extensive contribution for the revision of the manuscript.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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10. Published versions of selected publications

Chronically Epileptic Human and Rat Neocortex Display a Similar Resistance Against Spreading Depolarization In Vitro

Anna Maslarova, Mesbah Alam, Clemens Reiffurth, Ezequiel Lapilover, Ali Gorji and Jens P. Dreier

Stroke. 2011;42:2917-2922; originally published online August 11, 2011;
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Neurobiol Dis. 2012 Dec;48(3):495-506. doi: 10.1016/j.nbd.2012.06.024. Epub 2012 Jul 7.

Peri-infarct blood-brain barrier dysfunction facilitates induction of spreading depolarization associated with epileptiform discharges.

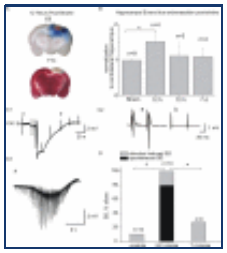
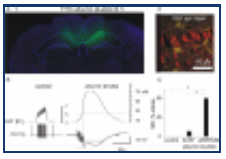
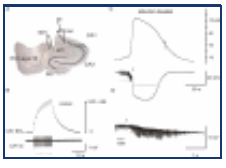
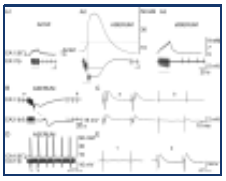
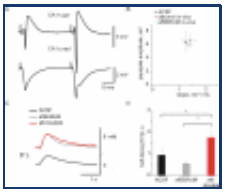
Lapilover EG[1], Lippmann K, Salar S, **Maslarova A**, Dreier JP, Heinemann U, Friedman A.

Author information

Abstract

Recent studies showed that spreading depolarizations (SDs) occurs abundantly in patients following ischemic stroke and experimental evidence suggests that SDs recruit tissue at risk into necrosis. We hypothesized that BBB opening with consequent alterations of the extracellular electrolyte composition and extravasation of albumin facilitates generation of SDs since albumin mediates an astrocyte transcriptional response with consequent disturbance of potassium and glutamate homeostasis. Here we show extravasation of Evans blue-albumin complex into the hippocampus following cortical photothrombotic stroke in the neighboring neocortex. Using extracellular field potential recordings and exposure to serum electrolytes we observed spontaneous SDs in 80% of hippocampal slices obtained from rats 24 h after cortical photothrombosis. Hippocampal exposure to albumin for 24 h through intraventricular application together with serum electrolytes lowered the threshold for the induction of SDs in most slices irrespective of the pathway of stimulation. Exposing acute slices from naive animals to albumin led also to a reduced SD threshold. In albumin-exposed slices the onset of SDs was usually associated with larger stimulus-induced accumulation of extracellular potassium, and preceded by epileptiform activity, which was also observed during the recovery phase of SDs. Application of ifenprodil (3 μM), an NMDA-receptor type 2 B antagonist, blocked stimulus dependent epileptiform discharges and generation of SDs in slices from animals treated with albumin in-vivo. We suggest that BBB opening facilitates the induction of peri-infarct SDs through impaired homeostasis of K+.

PMID: 22782081 [PubMed - indexed for MEDLINE] PMCID: PMC3588590 **Free PMC Article**

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Increased susceptibility to acetylcholine in the entorhinal cortex of pilocarpine-treated rats involves alterations in KCNQ channels.

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Abstract

In models of temporal lobe epilepsy, in-vitro exposure of the entorhinal cortex (EC) to low concentrations of acetylcholine (ACh) induces muscarinic-dependent seizure-like events. Potassium channels from the KCNQ/Kv7 family, which close upon activation of muscarinic receptors, are mutated in several epileptic syndromes such as benign familial neonatal convulsions (KCNQ2/KCNQ3) and sudden unexplained death in epilepsy (KCNQ1). Therefore, we tested the hypothesis whether the ictogenic effect of ACh involves alterations of KCNQ channels. In horizontal temporo-hippocampal slices from pilocarpine-treated chronically epileptic rats, field potential recordings of epileptiform activity were performed in response to the application of ACh, the KCNQ blocker linopirdine, and KCNQ agonists. In the EC of control rats, ACh (20 and 50 μM) induced nested fast activity in the range of 15-20 Hz riding on <1 Hz slow oscillations. By contrast, in slices from pilocarpine-treated rats, 5 μM ACh was sufficient to induce interictal discharges that frequently transformed to epileptiform events at 20 μM ACh. While the non-specific KCNQ/Kv7 channel blocker linopirdine (20 and 50 μM) had no effect in control animals, in slices from epileptic rats it induced interictal discharges or seizure-like events. These could be blocked by the unspecific KCNQ/Kv7 agonist retigabine and attenuated by the Kv7.1 agonist L364-373. Immunohistochemistry revealed reduced expression of KCNQ2 and KCNQ3 in the EC and of KCNQ3-positive dendrites in the subiculum of epileptic rats. These results indicate that channels of the KCNQ family are key regulators of seizure susceptibility and their decreased availability in the epileptic tissue may reduce seizure threshold and contribute to ictogenesis.

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14. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

15. Complete list of publications

Publications:

Maslarova A, Alam M, Reiffurth C, Lapilover E, Gorji A, Dreier JP:*Chronically epileptic human and rat neocortex display a similar resistance against spreading depolarization in vitro*. Stroke 2011 Oct; 42(10):2917-22.

Maslarova A, Salar S, Lapilover E, Friedman A, Veh R and Heinemann U: *Increased susceptibility to acetylcholine in entorhinal cortex of pilocarpine treated rats involves alterations in expression of KCNQ channels*. Neurobiol Dis. 2013 Aug;56:14-24

Lapilover EG, Lippmann K, Salar S, **Maslarova A**, Dreier JP, Heinemann U and Friedman A: *Peri-infarct Blood-Brain Barrier Dysfunction Facilitates Induction of Spreading Depolarization Associated with Epileptiform Discharges.* Neurobiol Dis. 2012 Dec; 48(3):495-506.

Klaft ZJ, Schulz SB**, Maslarova A**, Gabriel S, Heinemann U and Gerevich Z: *Extracellular ATP differentially affects epileptiform activity via purinergic P2X7 and adenosine A(1) receptors in naive and chronic epileptic rats.* Epilepsia. 2012 Nov;53(11):1978-86.

Submitted:

Maslarova A, Salar S, Lippmann K, Klaft ZJ, Hollnagel JO, Rösler A* and Heinemann U*: *Mechanisms underlying spontaneous subicular sharp waves in mice hippocampal slices,* submitted to Neuron, November 2013

Salar S, Weissberg I, Sheintuch L**, Maslarova A**, Lippmann K, Nichtweiss J, Kunz WS, Shorer Z, Friedman A and Heinemann U: *Blood-brain barrier dysfunction can contribute to pharmacoresistance of seizures,* submitted to Annals of Neurology, September 2013

Manuscripts in Preparation:

Maslarova A, Lippmann K, Salar S, Klaft ZJ and Heinemann U: *Spontaneous subicular field potential transients in slices from normal and kainate-treated mice*.

Selected Talks:

4th Annual Epicure Meeting, Marseille 2010: *Changes in expression of M channels in models of temporal lobe epilepsy.*

SFB/TR3 Junior Scientists Meeting 2010, Bonn*: A Kv7 channel phenotype in the kainate model of temporal lobe epilepsy.*

Berlin Brain Days 2010: *How does the M-type potassium channel affect excitability in the epileptic entorhinal cortex?*

Posters :

Maslarova A, Alam M and Dreier JP: *Nitric oxide synthase inhibition decreases the potassium threshold of cortical spreading depolarization in human and rat neocortical tissue.* FENS Abstr., vol.4, 184.39, 2008.

Maslarova A, Andrioli A, Wahab A, Derst C, Veh R and Heinemann U: *A Kv7 channel phenotype in the chronically epileptic entorhinal cortex.*; Society for Neuroscience, 2010. Poster350.29/M9.

Maslarova A, Salar S, Lippmann K and Heinemann U: *Pharmacological properties of spontaneous sharp waves in the mouse subiculum in vitro.* FENS 2012 Poster 141.23/B54.

Salar S, Lapilover E, **Maslarova A**, Lippmann K, Friedman A and Heinemann U: *The effects of compromised blood brain barrier on plasticity changes and response to antiepileptic drugs in the hippocampus.* FENS 2012 Poster 144.06/B116.

Lippmann K, Nichtweiß J, Reichert A, Bar-Klein G, **Maslarova A**, Heinemann U and Friedman A: *Altered Synaptic Plasticity And Rhythmic Oscillations In The Hippocampus Following Vascular Injury And Blood Brain-Barrier Dysfunction* FENS 2012 Poster 75.22 /B22

Klaft ZJ, Schulz SB, **Maslarova A**, Gabriel S, Heinemann U and Gerevich Z: *Endogenously activated P2X4, P2X7 and P2Y1 receptors have minor proconvulsive effects on epileptiform activity in rat medial entorhinal cortex* FENS 2012 Poster 34.15/C94.

Maslarova A, Salar S, Lippmann K, Klaft ZJ, Rösler A and Heinemann U: *Mechanisms underlying subicular sharp wave-like events.* Symposium Ion Channels in Health and Disease, University of Cambridge 2013

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